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Presence of Microorganisms in Hemolymph of the Horseshoe Crab *Limulus polyphemus*

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Hemolymph samples obtained from *Limulus polyphemus* at the time of collection and after a 1-week holding period exhibited a significant increase in bacterial levels. No differences were observed in the ability of amoebocyte lysate, prepared from these same samples, to gel in the presence of lipopolysaccharide.

There is a range of findings with regard to bacteria in invertebrate blood systems. Hemolymph from sipunculid worms has been shown to be sterile (F. B. Bang and S. M. Krassner, Biol. Bull. [Woods Hole] **115**:343, 1958), as has that of the California sea hare (9). On the other hand, over 20% of the hemolymph samples from lobsters reportedly contained bacteria (12). These studies utilized a selective primary medium which may have reduced the number of bacteria isolated. Perhaps the most extensive study on the bacteriology of hemolymph was carried out with the blue crab. Examining 290 hemolymph samples, Tubiash et al. (13) found that 18% of them were sterile. The mean most probable number (MPN) per milliliter of hemolymph was 2,756 for males and 1,300 for females.

The hemolymph of the American horseshoe crab *Limulus polyphemus* contains several lectins (2, 7). Cells from this animal are the souce of the amoebocyte lysate, used to detect lipopolysaccharide (LPS) (6). Marked variability has been found among batches of amoebocyte lysate in terms of their ability to detect endotoxin (4). The present study was designed to examine *L. polyphemus* hemolymph for the presence of bacteria as a function of holding time of these animals after collection. A long-term goal is to assess the effects of such a microflora on the products derived from *L. polyphemus*.

For each experiment, animals were bled aseptically, once shortly after collection then again after 1 week of maintenance in flowing estuary water tanks (temperature range, 15 to 25°C). Animals with overt signs of trauma (e.g., missing appendages) were excluded from this study. For estimation of bacterial numbers, hemolymph samples were obtained by cardiac puncture. One milliliter of hemolymph was added to 9 ml of 2216E medium (8); further dilutions, in the same medium, were made from this inoculated broth. Hemolymph samples from the initial bleeding were tested at 10^0 and 10^{-1} dilutions, and those obtained after holding were additionally diluted to 10^{-2} and 10^{-3}. Each sample was tested in duplicate at each stated dilution. Growth was determined by turbidity in the tubes after incubation at 18°C for 5 days. Information from these studies was used to compute MPN values by conversion of the raw values to scores based on a three-tube-per-dilution schema (1).

Concomitant with the bacteriological studies, individual hemolymph samples were obtained for preparation of amoebocyte lysate. Ten milliliters of hemolymph was collected directly into 15 ml of a buffered, isotonic solution of *N*-ethylmaleimide. The cells were washed, lyophilized, and ground with a glass rod before being suspended in lysate buffer (14). For each assay, 20 μl of the lysate sample to be tested was mixed with an equal volume of the appropriate dilution of LPS (Associates of Cape Cod, Woods Hole, Mass.) on a pyrogen-free, siliconized glass slide. Twofold dilutions of LPS ranging from 1 μg ml^{-1} to 125 ng ml^{-1} were tested. Each mixture was drawn into a 100-μl micropipette, care being taken to eliminate any air bubbles at the pipette tip. The filled pipettes were incubated horizontally in a moisture chamber for 45 min at 37°C. One drop of nigrosin was then placed at the sample end of the micropipette and the pipette was inverted (sample end up) to allow penetration of the dye into the test mixture. Failure of the dye to penetrate was interpreted as a positive lysate test.

Under the conditions employed in these studies, most of the hemolymph samples contained no detectable bacteria (73% of samples from the initial bleeding and 59% from the 1-week bleeding). Of the 20 hemolymph samples from the initial bleeding found to contain bacteria, only 2 (10%, or 2.7% of the total number of samples at this bleeding) had MPN values >20. In the samples obtained from the 1-week bleedings, 17 of 31 positive samples (55%, or 24% of the total number of samples collected at this time) had MPN values >20, and 8 (26%, or 11% of the total number of samples collected at this time) yielded MPN values >100.

For the lysate studies, 13 sets of lysate samples, i.e., samples collected from 13 horseshoe crabs at $t = 0$ and from the same animals at $t = 1$ week, were obtained from animals with no detectable bacteria flora and similar sets of lysate samples were obtained from those with positive flora. Eleven of 26 samples (42.3%) from animals with detectable bacteria in their hemolymph yielded positive lysate activity. Among the samples examined from crabs with no detectable bacteria in their hemolymph, 6 of 26 (23%) yielded positive lysate activity. The results were analyzed by the use of two-tailed chi-square tests for independent observations and for corre-

TABLE 1. Statistical analyses with the chi-square test

System tested	χ^2	df	*P*
Change in bacterial level of hemolymph over time	14.06	1	<0.005
Bacterial flora in hemolymph vs lysate activity	2.12	1	>0.10
Lysate activity vs time of collection	0	1	1.0
Lysate activity vs sex	1.42	1	>0.20
Hemolymph flora vs sex at t_0	2.03	1	>0.10
Hemolymph flora vs sex at t_1	0.908	1	>0.30

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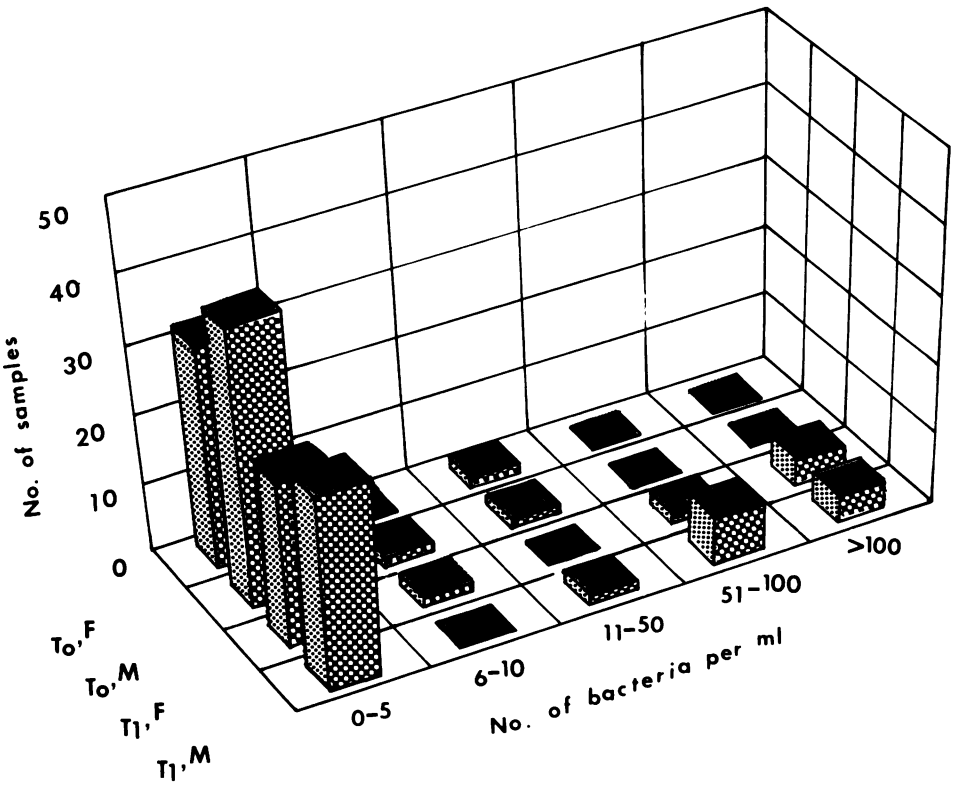

FIG. 1. Distribution of hemolymph samples collected from male and female horseshoe crabs at time of collection (t_0) and 1 week later (t_1), with respect to bacterial levels.

lated data (5; Table 1). The lysate activity of the sample collected associated with none of the other variables examined: time of collection, presence of bacteria in the hemolymph, or sex of the animals studied. Similarly, the presence of detectable bacteria in the hemolymph samples did not associate with lysate activity or sex of the animals. When the number of hemolymph samples with low or undetectable levels of bacteria (<50 organisms ml^{-1}) versus those with levels exceeding this value at t_0 were compared with similar data groups at t_1, a significant difference ($P < 0.005$) was found (Fig. 1).

These findings indicate that horseshoe crabs, like other marine invertebrate species that have been examined to date, may contain microorganisms in their hemolymph. Huq et al. (3) have suggested that copepod-microbe associations may be mutually beneficial. It is possible that microorganisms found in fluid samples such as hemolymph may not reflect those organisms in intimate association with the host, since the latter are firmly adhered to tissues (3). Whether such an adherent population of microorganisms is present in *L. polyphemus* is not known.

The presence of microorganisms in the hemolymph did not seem to interfere with the activity of amoebocyte lysate obtained from these animals. This finding was unexpected, since the lysate is reactive with LPS from gram-negative bacteria and virtually all of the isolates obtained from these hemolymph samples were gram-negative bacilli (E. R. Brandin, unpublished data). Limulin, a lectin from *L. polyphemus* which reacts with 2-keto-3-deoxyoctonate (11), a core sugar in many forms of LPS, agglutinates a wide variety of gram-negative bacteria, but there are some exceptions, e.g., *Serratia marcescens* (10). It would be interesting to determine whether the hemolymph isolates or their extracted LPS are reactive with *Limulus* amoebocyte lysate.

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